EFFECT OF CAULIFLOWER MOSAIC VIRUS INFECTION ON THE METHYLATION OF ARABIDOPSIS THALIANA DNA

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LIST OF ABBREVIATIONS

A	Adenine
ADH	Alcohol dehydrogenase
azaC	5-Azacytidine
bp	Base pair (s)
BSA	Bovine serum albumin
C	Cytosine
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
d	Deoxy
DNA MTase	DNA (cytosine-5)-methyltransferase
EDTA	Ethylenedinitrilo-tetraacetic acid disodium salt
G	Guanine
Kb	Kilo base pair (s)
5-mC	5-Methylcytosine
mRNA	Messenger RNA
^{32}P	Radioisotope of phosphorus
PCR	Polymerase chain reaction
PEPCase	Phosphoenolpyruvate carboxylase
rDNA	Ribosomal DNA
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate

T	Thymine
UV	Ultraviolet
V	Volts
vol	Volume

CHAPTER I

INTRODUCTION

DNA methylation in plants is important in gene regulation. Changes in the methylation status of genes can alter gene expression and cell functions. Methylation of DNA *in vitro* inhibited transcription in regenerated plants when the DNA was stably integrated (Weber et al., 1990), as well as transiently in protoplasts (Hershkovitz et al., 1989; Weber and Graessmann, 1989). In plants, some DNA-binding proteins no longer recognized their target site *in vitro* if one or more cytosine residues within the site were methylated (Gierl et al., 1988; Staiger et al., 1989; Inamdar et al., 1991). As some of these DNA-binding proteins could be transcription factors, *in vivo* methylation of their binding sites may inhibit transcription. DNA methylation also affected chromatin structure, which was reflected by changes in the sensitivity of intact chromatin to DNase I; in particular, sensitivity of chromatin to DNase I was highly associated with hypomethylated DNA (Klaas and Amasino, 1989). Methylation of DNA could suppress transcription either directly by disrupting the binding of transcription factors, or indirectly when binding of methyl-binding proteins to the DNA or changes in chromatin structure makes a gene inaccessible for transcription.

The expression of some plant genes was correlated with specific and regulated changes in methylation in the vicinity of the genes (Bianchi and Viotti, 1988; Spena et al., 1983; Flavell and O'Dell, 1990; Riggs and Chrispeels, 1990; Langdale et al., 1991; Flavell et al., 1990; Kaufman et al., 1987). Changes in the methylation patterns occur during normal plant development ; for instance, the methylation of Zea mays *Spm* transposable elements

was not constant throughout the life cycle (Fedoroff et al., 1989). Hypermethylation of the DNA sequence within promoter regions can inhibit gene expression. The extent to which changes in methylation are associated with gene regulation can be revealed by methylation-sensitive restriction patterns and/or genomic sequencing.

Environmental stimuli can induce changes in the methylation status of DNA. The overall level of DNA methylation in cotton plants was altered by fungal infection (Guseinov et al., 1975). The vernalization induced by cold to promote flowering was correlated with changes in DNA methylation (Lang, 1965). Alteration of the expression of plant genes by virus infection could lead to visible symptoms of viral disease of the plants. Methylation of barley DNA was greatly reduced by infection with either brome mosaic virus or barley streak mosaic virus (Vanyushin et al., 1971).

A starting point for investigating the methylation status of the plant genome is to examine methylation-sensitive restriction patterns of the plant DNA. The aim of this study is to determine whether the methylation-sensitive restriction patterns of Arabidopsis thaliana DNA are altered by infection with cauliflower mosaic virus (CaMV). Digestion of the plant DNA with restriction endonucleases sensitive to specific methylated-cytosine residues and Southern blot-hybridization were used to examine possible differences in methylation patterns by comparing the sizes of fragments generated from the DNAs of CaMV-infected and healthy plants.

There were no significant differences in the methylation patterns of fragments from healthy and CaMV-infected A. thaliana DNA, suggesting that, at least for this virus-host combination and for the genes probed and restriction sites examined, changes in DNA methylation probably play no role in the generation of disease symptoms. Nevertheless, as a very limited number of the potential methylation sites in A. thaliana DNA could be assayed by using methylation-sensitive restriction enzymes, the failure to detect specific changes in DNA methylation in A. thaliana genome by CaMV infection does not necessarily imply that DNA methylation is not involved in gene regulation.

CHAPTER II

LITERATURE REVIEW

Properties of DNA Methylation

Plant DNA contains the modified base 5-methylcytosine (5-mC). In plant cells, up to 30% of cytosine residues were methylated (Adams and Burdon, 1985), while only 3-8% of cytosine residues in mammalian cells were modified (Shapiro, 1975). The gross level of cytosine methylation in plant genomes was quite different, ranging from 4.6% in Arabidopsis thaliana (Leutwiler et al., 1984), with a small genome size and relatively little highly repeated DNA sequences, to 33% in rye, Secale cereale (Thomas and Sherratt, 1956). The methylation of cytosine residues in plant DNA takes place specifically at CpG and CpNpG sequences, where N can be any base (Gruenbaum et al., 1981). When modified, both the CpG and CpNpG sequence motifs have symmetrically methylated cytosine residues in the DNA double strands, which allows the patterns of methylation to be maintained through DNA replication (Cedar et al., 1979).

Cytosine methylation is catalyzed by the enzyme DNA (cytosine-5)-methyltransferase (DNA MTase), which transfers methyl groups to cytosine residues at position 5 from S-adenosyl methionine (Adams and Burdon, 1983). The DNA MTases from pea shoots, wheat embryos and cultured rice cells have been purified (Yesufu et al., 1991; Theiss et al., 1987; Giordano et al., 1991). Specifically, the pea DNA MTase modified cytosine residues in all four dinucleotides (CA, CC, CG and CT), suggesting that both CpG and CpNpG

motifs could be modified. Further, the level of CG methylation was higher than that of the other dinucleotides, reflecting methylation of CG in addition to CGG (Yesufu et al., 1991).

The distribution of 5-mC in plant genomes is far from random. First, as mentioned early, cytosine methylation in plants takes place exclusively at CpG and CpNpG sequence motifs (sequence control). Second, for tissue-specific genes in plants, demethylation of specific cytosine residues is required for transcriptional activation of the genes (tissue-specific control). 5-mC is present 5' of nonexpressed genes, while unmodified cytosine residues are found 5' of the expressed genes (reviewed in Yisraeli and Szyf, 1984). For example, for the maize A1 (dihydro-flavonol 4-reductase) gene, the CpG clusters within the coding region were unmethylated while other CpG motifs about 2kb upstream were methylated. The 5' CpG-rich regions of both maize Adh1 (alcohol dehydrogenase) and Sh1 (sucrose synthase) genes were not methylated nor were the neighboring coding sequences (Antequera and Bird, 1988). Third, the methylation status of cytosine residues in plant genomes is inherited in a programmed manner during cell development and differentiation (biological control). For instance, unique sequences, approximately 38% of the cotton genome, contain only 4% of methylated cytosine residues (Guseinov et al., 1975). Most of the cytosine methylation in A. thaliana DNA was present in the highly repeated DNA sequences, such as the 180-bp centromeric repetitive sequence and ribosomal RNA genes (Pruitt and Meyerowitz, 1986).

DNA Methylation versus Chromatin Structure

Methylation of cytosine residues affects the structure of chromatin (reviewed in Lewis and Bird, 1991) and affects the accessibility of DNA to endonucleolytic cleavage. In general, chromatin sensitive to DNase I has a lower level of DNA methylation than that of bulk chromatin. This correlation, however, is protein dependent. The sensitivity of chromatin to DNase I was no longer correlated with the level of DNA methylation, when naked DNA was isolated from chromatin (Klaas and Amasino, 1989). By comparison with

bulk chromatin, nucleosomes digested with restriction enzymes sensitive to ^{m}CpG motif had an approximately 10% reduced level of histone H1, and histones H3 and H4 were more acetylated in the digested nucleosomes than those in bulk chromatin (Lewis and Bird, 1991). Acetylated histones H3 and H4 have been associated with active chromatin, suggesting that hypomethylated DNA in chromatin was active (Hebbes et al., 1988). In addition, Spiker et al. (1983) indicated that DNase I-sensitive DNA was both hypomethylated and actively transcribed.

DNA Methylation Affects DNA Replication

DNA methylation may affect the temporal regulation of DNA replication during the cell cycle (reviewed in Lewis and Bird, 1991). In early embryogenesis, inactivation of one of the X chromosomes was concurrent with methylation of CpG dinucleotide, and with a shift of replication time of this inactive X chromosome to late in the S phase in the cell cycle (Grant and Chapman, 1988). Jablonka et al. (1985) provided evidence that methylation may play a role in the shift of replication time of the inactive X chromosome. When treated with 5-azacytidine (azaC), a potent inhibitor of DNA methylation, the inactive X chromosome became progressively demethylated and its replication time shifted to the early S phase, suggesting that alteration of methylation status of the X chromosome could lead to changes in its replication time. Further, in F9 embryonal carcinoma cells, the replication time of hypomethylated satellite DNA was also shifted to the early S phase (Selig et al., 1988). In addition, at the replication origin of E. coli, GATC sequence motifs were always methylated under normal growth conditions (Razin et al., 1980).

DNA Methylation Induces Spontaneous Mutation

DNA methylation can result in an increase in the frequency of spontaneous mutations. Cytosine residues were deaminated to uracil (Shapiro and Klein, 1966), which could be detected and removed from DNA by the enzyme uracil-specific N-glycosidase (Lindahl,

1974). On the other hand, deamination of 5-mC converts it to thymine. As thymine is a normal component of DNA, the mismatched T-G pair could be repaired either to a T-A pair or to a C-G pair in the absence of a directional repair system. The conversion of ^{m}C-G pair to T-G pair, due to deamination of 5-mC, may give an explanation for the observation that the T-G frequency in the genomes of both plants and animals was higher than expected (Bird, 1986). Nevertheless, in plant cells the frequency of CpNpG trinucleotides does not appear to be reduced by the deaminational loss in methylated DNA.

DNA Methylation Affects DNA-Protein Interactions

The primary effect of methylating cytosine residues in DNA is to modify or to alter the interaction of specific DNA sequences with a wide range of DNA-binding proteins. Basically, 5-mC introduces a methyl group into an exposed position in the major groove of the DNA helix. The binding of such DNA-binding proteins to DNA as the *lac* repressor, pea seedling histones, and estrogen receptor (E_2R) can be affected by an exposed methyl group in the major groove of DNA helix (Lin and Riggs, 1972; Lin et al., 1976; Kallos et al., 1978). For instance, when the thymine residue at nucleotide position 13 in the *lac* operator was changed to uracil or cytosine, the affinity of *lac* repressor for this operator greatly decreased; however, changing the position 13 from cytosine or uracil to 5-mC restored the affinity for *lac* repressor to normal (Fisher and Caruthers, 1979). Thus, the *lac* repressor only senses the presence or absence of the methyl group at the nucleotide position 13. Further, in the alternating polynucleotide poly(dG·dC)·poly(dG·dC), the replacement of cytosine with 5-mC resulted in the transition from the right-handed B form to the left-handed Z form DNA (Behe and Felsenfeld, 1981; Behe et al., 1981; Klysik et al., 1983). In contrast to the B form DNA stabilized by the formation of nucleosome core particles, Z form DNA could prevent the formation of nucleosomes (Nickol et al., 1982). Therefore, DNA methylation can affect both secondary structure of DNA itself and tertiary structure of the chromatin. Furthermore, the protein, tnpA, encoded by the most abundant transcript of

the maize transposable element *Spm (En1)* could bind to a repeated 12-bp sequence motif in the subterminal repetitive region of *Spm* ; binding of this protein was inhibited by methylation of cytosine residues present in the CCG trinucleotide on one strand of this 12-bp repeat sequence and in the corresponding CG dinucleotide of the complementary strand (Gierl et al., 1988).

DNA methylation can affect the accessibility of promoter sequences to transcription factors either directly or indirectly. The regional methylation of CpG-rich promoter sequences can repress transcription (Busslinger et al., 1983; Keshet et al., 1985; Lewis and Bird, 1991). The binding of promoter or enhancer sequences to transcription factors from mammalian cells can be inhibited by *in vitro* CpG methylation of these sequences (Shen and Whitlock, 1989; Comb and Goodman, 1990). Similarly, the binding of plant DNA-binding protein CG-1 isolated from tobacco nuclear extracts to a sequence containing a CACGTG motif in the promoter region of the Antirrhinum chalcone synthase gene was inhibited by *in vitro* methylation of the target sequence motif (Staiger et al., 1989). Further, a specifically methylated-DNA-binding protein MeCP, identified in mouse cells, binds preferentially to DNA sequences containing 15 or more 5-mC·G dinucleotides without other sequence specificity (Meehan et al., 1989). Binding of MeCP inhibited transcription from methylated promoters both *in vitro* and *in vivo* (Boyes and Bird, 1991). In addition, another methylated-DNA-binding protein MDBP, identified in pea nuclear extracts, could inhibit transcription when binding to specific sequence motifs (Zhang et al., 1989).

DNA Methylation Alters Gene Expression

DNA methylation is involved in the regulation of gene expression. The role of DNA methylation in regulation of gene expression in animal cells has been documented (reviewed in Razin and Cedar, 1991). The effects of DNA methylation on gene expression studied on the rabbit (Waalwijk and Flavell, 1978), chicken (McGhee and Ginder, 1979),

and human (van der Ploeg and Flavell, 1980) globin genes indicated that the globin genes were unmethylated in the tissue of expression but were methylated in DNA from other tissues. Methylation of a specific cytosine residue within the target site may inactivate the genes by preventing the binding of transcription factors (Inamdar et al., 1991); by contrast, methylation of cytosine residues outside the protein binding sites may have no effect on gene activity. The current model proposes that undermethylation of specific DNA sequences within the promoter region is a prerequisite for gene expression. For example, hypo-methylation of wheat rDNA has been correlated with active rDNA units (Flavell et al., 1988; Kaufman et al., 1987), and the promoter region is unmethylated when the gene transcribed. Similarly for the *Ac* transposable element, there was a clear correlation between the *Ac* activity and hypomethylation of the 5' region upstream of the initiation codon of the transposase protein (Schwartz and Dennis, 1986). Further, the expression of rat growth hormone gene was correlated with an unmethylated CGCG sequence near the transcriptional initiation site (Strobl et al., 1986). Direct evidence that DNA methylation can block gene expression has been obtained by gene transfer experiments (Wigler et al., 1981; Buschhausen et al., 1985).

5-Azacytidine Induces DNA Demethylation

The DNA methylation inhibitor, 5-azacytidine (azaC), has been used both to induce DNA demethylation and to alter gene expression (Jones, 1985; reviewed in Razin and Cedar, 1991). AzaC is incorporated into DNA during DNA replication or repair (Taylor et al., 1984), and then the DNA MTase activity can be inhibited by azaC (Taylor and Jones, 1982). Treatments with azaC have resulted in a clear correlation between the decrease in the 5-mC content and the increase in the level of expression of genes, such as the *rbc* L gene, growth hormone gene and induced isopentenyl transferase gene (Ngernprasirtsiri and Akazawa, 1990; Lan, 1984; Klaas et al., 1989). Further, the inactive endogenous retrovirus gene in chicken AEV cells was turned on after the cells were exposed to azaC,

and demethylation of the gene sequence was verified (Groudine et al., 1981). In addition, azaC could induce the reactivation of a silent, introduced ß-glucuronidase (GUS) gene driven by the CaMV 35S promoter in the transgenic Nicotiana tabacum tabacco plant (Bochardt et al., 1992).

DNA Demethylation and Gene Expression

Activation of tissue-specific genes is often accompanied by the demethylation of cytosine residues from promoter sequences. Housekeeping genes contain a CpG-rich region at their 5' end, and this region is completely unmethylated in all tissues upon expression of the genes (Bird, 1986; Stein et al., 1983). Langdale et al. (1991) reported that specific cytosine demethylation was correlated with tissue-specific and light-regulated expression of the maize PEPCase gene. In this case, demethylation of a single PvuII site located approximately 3.3 kb upstream of the transcription start site of the PEPCase gene was related to the accumulation of PEPCase mRNA during greening. Demethylation at this site was restricted to mesophyll cells, where the PEPCase gene was expressed. The PvuII recognition sequence is CAGCTG, thus suggesting that methylation of CpNpG trinucleotides, specifically ^{m}CTG motif, as well as CpG dinucleotides is important in gene regulation (Langdale et al., 1991). In addition, for light-induced rRNA synthesis in pea, demethylation of HpaII sites within the non-transcribed spacer (NTS) region of pea rDNA could result in a two-fold increase of rRNA synthesis (Gallagher and Ellis, 1982; Kaufman et al., 1987).

DNA Methylation versus Environmental Stimuli

The methylation status of DNA alters in response to environmental stimuli. Infection of cotton plants with the fungus Verticillium dahliae resulted in a decreased level of DNA methylation of the plant (Guseinov et al., 1975); in particular, cytosine methylation in highly repeated DNA sequences was decreased about three fold, while almost no

significant changes of cytosine methylation occurred in unique sequences. Similarly, methylation of barley DNA was greatly reduced by infection with either brome mosaic virus or barley streak mosaic virus (Vanyushin et al., 1971). Further, virus infection can alter the expression of host genes. Infection of murine thymocytes with radiation leukemia virus resulted in the reduced expression of Class I antigens and the concomittant increased methylation of the MHC (major histocompatibility complex) genes (Meruelo et al., 1986). In addition, changes in DNA methylation were correlated with vernalization which is the promotion of flowering by cold (Lang, 1965). Changes in the methylation status of genes involved in floral initiation has been implicated in the vernalization response of late flowering ecotypes of Arabidopsis (Burn, 1993). Peschke et al. (1991) have proposed that altered status of DNA methylation in response to environmental changes may result from changes in the activity of the maintenance enzymes involved in DNA methylation and changes in DNA replication and cell division, or could be related to chromosome breakage and repair as occurred in cultured cells.

Arabidopsis thaliana versus CaMV Infection

A. thaliana is a prolific cruciferous plant with a relatively small genome size and little dispersed repetitive DNA sequences in its genome (Pruitt and Meyerowitz, 1986; Meyerowitz, 1987). A. thaliana is a host for CaMV, a well characterized caulimovirus, which contains an 8-kp double-stranded circular DNA as its genetic material (Covey, 1985; Maule, 1985). In the CaMV DNA, the content of CpG dinucleotide is low (Russell et al., 1971). For the virus-host interaction, the replication of CaMV in A. thaliana causes disease symptoms. These disease symptoms have been described (Balazs and Lebeurier, 1981; Melcher, 1989), and include vein clearing, chlorotic spotting, and stunting. The disease symptoms may result from changes in the gene expression of A. thaliana , and the changes in gene expression may be influenced by cytosine methylation or demethylation in A. thaliana DNA, which could be induced by CaMV infection. In brief, the combination of A.

thaliana and CaMV is a simple system for studying the relationship between changes in cytosine methylation of the plant DNA via virus infection and visible disease symptoms of the plant.

Arabidopsis thaliana DNA Methylation ddm1 Mutants and 180-bp Centromeric Repeat

Vongs et al. (1993) isolated three A. thaliana DNA hypomethylation ddm1 mutants. In the genomic DNA of these ddm1 mutants, the amount of 5-mC was globally reduced over 70 percent at both CpG and CpNpG sites, and the centromeric repetitive arrays were uncharacteristically susceptible to the restriction digestion by a methylation-sensitive restriction enzyme HpaII or MspI. Hybridization of a nylon membrane carrying the HpaII-digested fragments of both wild-type A. thaliana DNA and the hypomethylation mutant DNAs with the A. thaliana 180-bp centromeric repetitive sequence showed on an autoradiograph, for the A. thaliana mutant DNAs rather than wild-type A. thaliana DNA, a series of regularly spaced bands of lower molecular weight, implying that the A. thaliana centromeric repetitive DNA arrays were highly hypomethylated in these ddm1 mutants. Therefore, the question raised here is whether or not the methylation status of cytosine residues in the A. thaliana centromeric repetitive DNA arrays could be altered by CaMV infection.

Methods for Studying DNA Methylation

Gross changes in DNA methylation can be assessed by determining the frequency of 5-mC in total DNA. Methylation-sensitive restriction enzymes can be used to determine the methylation status of specific cytosine residues in the recognition site. Any change in the size of fragments generated can be detected by Southern blot-hybridization using appropriate probes. However, only a few of all potentially methylated sites of a given genome can be assayed by this method (Winnacker, 1984). For instance, the methylation status of CC(A/T)GG motif can be examined by using isoschizomers ApyI, EcoRII and

BstNI, all of which recognize their target sequence but differ in their ability to cleave the methylated DNA. Though both methylation-sensitive restriction enzyme HpaII and MspI can not cleave the methylated sequences ^{m}CCGG and ^{m}C^{m}CGG, internal cytosine-methylated C^{m}CGG sequence can be cleaved by MspI rather than HpaII (Kessler et al., 1985). Nevertheless, the methylation sites that do not lie within a restriction enzyme recognition site can not be examined by this method. Moreover, this method has the severe drawback that hemimethylation often remains undetected (Gruenbaum et al., 1981).

Genomic sequencing, which distinguishes between modified and unmodified cytosine residues in DNA because of their chemical nature, can be used to detect the methylation status of cytosine residues that are not within a restriction site (Church and Gilbert, 1984). A refinement of this technique with increase in sensitivity, based on PCR amplification of fragments generated by chemical cleavage of DNA at unmethylated cytosine residues, has been reported (Saluz and Jost, 1989; Pfeifer et al., 1989). Further, bisulphite treatment of genomic DNA converts unmethylated cytosines to uracils, while 5-mC is not affected. The treated DNA is then amplified by PCR and sequenced, such that uracils will be replaced by thymines and the remaining cytosine residues in the DNA sequence must correspond to a methylated cytosine in the starting DNA (Frommer et al., 1992).

CHAPTER III

MATERIALS AND METHODS

Growth of Arabidopsis thaliana Plants

Seeds of A. thaliana cv. Columbia, a gift from Dr. David Meinke (Department of Botany, Oklahoma State University), were surface-sterilized successively by using 70% conc. ethanol (EtOH) for 60 sec. and 50% conc. bleach solution for 6 min. (final conc. 2.73% sodium hypochlorite [w/w] with 1 drop Tween 20). Dark green plastic pots with 3 or 4 inch diameter, individually fitted with 2 layers of Kimwipe and approximately 1 cm of small-grain perlite (Nord) on the bottom, were filled with the Soil Mix (12 parts of vermiculite (Strong-Lite), 3 parts of potting soil (Redi-Earth) and 1 part of sterilized sand (Handi-Sak)). The seeds were then planted on the surface of dry Soil Mix (6 seeds well separated on each pot and 12 pots in one dishpan), and watered with a fine, light mist until the surface of Soil Mix was very damp and the dishpan surface was wet. The dishpan was covered with clear plastic wrap to keep the humidity high. These A. thaliana plants grew at room temperature under fluorescent lamps. The pots were misted with water 2-3 times per day until seed germination. The plastic wrap was removed over 1-2 days by perforating it in a few places and folding it back at the corners of the dishpan when the cotyledons emerged. Then, I used a wash bottle to top-water around and between the seeds. After the first true leaves appeared, I used half-strength AN solution (one gallon half-strength AN solution contained 3.0 gm Hyponex and 0.2 gm Peters) to bottom-water the plants for about 20 min. once a day for 4 or 5 days. Thereafter, the plants were bottom-watered with full-strength AN solution (one gallon full-strength AN solution contained 6.0 gm Hyponex and 0.4 gm Peters) until the completion of plant growth.

Virus Infection

CM4-184, an isolate of CaMV (Pirone et al., 1960), was used to infect 3-week old A. thaliana plants. Virus infection of A. thaliana plants was accomplished by gently rubbing a 10 µl drop of the 0.2% (µg/µl) conc. viral inoculum containing 1 mg/ml Celite and 1% K_2HPO_4 on the surface of plant leaves (3 leaves for one plant) by a pipet tip. Healthy A. thaliana plants without mock inoculation were used as controls. Both the virus-infected and healthy A. thaliana plants grew at room temperature under fluorescent lamps kept on a 16 hr-light and 8 hr-dark period. All the plants were bottom-watered once per day with the full-strength AN solution. When symptoms appeared on the plant leaves (approximately 2-3 weeks after virus inoculation), the sick and healthy leaf tissues were taken off and put in 0.15 gm aliquots into 1.5 ml Eppendorf tubes. These Eppendorf tubes were then frozen by dipping and filling in liquid nitrogen. After being ground well with a small pestle, these leaf tissues in the Eppendorf tubes were stored in a -70 °C freezer for later plant DNA extraction.

Extraction of Plant DNA

The DNAs from the leaf tissues of both CaMV-infected and healthy A. thaliana plants were respectively extracted by following the preparation method "Plant DNA Mini-Prep" modified by Doug Dahlbeck (From Tai and Tanksley, 1991). In each Eppendorf tube having the plant leaf tissues collected and frozen as described above, 0.7 ml of the extraction buffer preheated to 65 °C, containing 100 mM Tris-HCl, pH 8.0, 50 mM EDTA, pH 8.0, 500 mM NaCl, 1.25% conc. SDS (w/v), 8.3 mN NaOH and 0.38% conc. Na bisulfite, was added and mixed thoroughly with a pipet tip. The Eppendorf tubes were put into a 65 °C water bath for 10 min. Then, 0.22 ml of 5 M potassium acetate was added into each tube and mixed well; the tubes were kept on ice for 30 min. The tubes were centrifuged at 10,000 rpm at 4 °C for 3 min. to pellet the precipitates. For each tube, the

supernatant was filtered through a small Kimwipe and the filtrate was collected into a new 1.5 ml Eppendorf tube, to which 0.7 vol. of isopropanol was added. The tubes were centrifuged at 10,000 rpm at 4 ^{o}C for 3 min. to pellet the precipitates. For each tube, the supernatant was discarded, and the precipitate was rinsed twice with 70% conc. EtOH and drained for 1 min. Three hundred µl T5E buffer (50 mM Tris-HCl, pH 8.0 and 10 mM EDTA, pH 8.0) was added into each tube; the tubes were vortexed for 2 sec., put into a 65 ^{o}C water bath for 5 min., and then vortexed for 2 sec. again to make sure that the precipitates were resuspended. For each tube, 150 µl of 7.5 M ammonium acetate was added. The tubes were vortexed for 2 sec., and centrifuged at 10,000 rpm at 4 ^{o}C for 3 min. to pellet the precipitates. For each tube, the supernatant was transfered into a new 1.5 ml Eppendorf tube, to which 330 µl isopropanol was added and then mixed well. Again, the tubes were centrifuged at 10,000 rpm at 4 ^{o}C for 3 min. to pellet the precipitates. For each tube, the supernatant was discarded, and the precipitate was rinsed twice with 70% conc. EtOH and then drained for 2 min. One hundred µl T5E buffer was added into each tube; the tubes were vortexed for 2 sec., put into a 65 ^{o}C water bath for 5 min., and then vortexed for 2 sec. For each tube, 10 µl of 3 M sodium acetate and 75 µl of isopropanol were added and mixed well. The tubes were centrifuged at 10,000 rpm at 4 ^{o}C for 3 min. to pellet the precipitates. For each tube, the supernatant was discarded, and the precipitate was rinsed twice with 70% conc. EtOH and drained for 2 min. For drying the precipitates, the tubes were centrifuged in Speedvac for 15 min. Then, 25 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) was added into each tube; the tubes were stored in a 4 ^{o}C refrigerator overnight to let the precipitates resuspend. Finally, the tubes were vortexed for 2 sec., put into a 65 ^{o}C water bath for 5 min., and then vortexed for 2 sec. These tubes containing the A. thaliana plant DNA were stored in a 4 ^{o}C refrigerator. The amount of plant DNA extracted was determined by DNA Fluorometer (Model TKO 100, Hoefer Scientific Instruments, San Francisco; Fluorescent dye: Hoechst 33258, bis-benzimidazole) or Spectrophotometer (Shimadzu UV160U, Japan; A_{260}/A_{280}).

Isolation of Plasmid DNA

Plasmid pGEMadh1 (in host, DH5α), containing a 3.6 Kb HindIII fragment with part of the A. thaliana alcohol dehydrogenase (ADH) gene (5' end, 2.6 kb fragment), was constructed by Dr. R. Pennington (Pennington's Ph.D. dissertation, 1991, Oklahoma State University) from the 3.6 Kb HindIII fragment of the ADH gene cloned in λAt3101 (Chang and Meyerowitz, 1986). Plasmid pSRL51 (from plasmid pUC119 in host, E. coli MV1190) containing part of the A. thaliana α-tubulin gene (3' end, 1.2 kb HindIII fragment ; Ludwig et al., 1987) was obtained from Dr. D. P. Snustad (University of Minnesota). Plasmid p5930MR (from pGEM3Z in host, DH5α) containing an A. thaliana 180-bp centromeric repetitive fragment was obtained by cloning random HindIII fragments of A. thaliana DNA into pGEM3Z (Maryam Rafie-Kolpin, Department of Biochemistry, Oklahoma State University, unpublished). The plasmid DNAs, containing the A. thaliana ADH gene, α-tubulin gene, or 180-bp centromeric repetitive fragment, were isolated by applying the commerical reagent kit "QIAGEN Plasmid Reagent" (QIAGEN Inc., Studio City, CA). Two hundred and fifty ml TB broth contained 6 gm yeast extract, 3 gm tryptone, 1 ml glycerol and 225 ml water (autoclaved and cooled), plus 25 ml of sterile TB Phosphate (0.17 M KH_2PO_4 and 0.72 M K_2HPO_4) and 3.1 ml ampicillin (5mg/ml). The colonies individually containing the plasmid DNA were inoculated in the 250 ml TB broth and grown at 37 °C with shaking for 36-48 hours. The cells were harvested by centrifugation at 8000 rpm for 10 min. The protocol supplied with the QIAGEN kit was followed. The cell pellets were resuspended in 10 ml of reagent P1 (100 μg/ml RNase A, 50 mM Tris-HCl, and 10 mM EDTA, pH 8.0), and 10 ml of reagent P2 (200 mM NaOH and 1% SDS) was added and mixed gently. The samples were incubated at room temperature for 5 min. Then, 10 ml of chilled reagent P3 (3 M potassium acetate, pH 5.5) was added and mixed immediately but gently, and the samples were incubated on ice for 20 min. The samples were centrifuged at 30000 xg at 4 °C for 30 min., and then the

supernatants were removed promptly. The column "QIAGEN-tip 500" was previously equilibrated by applying 10 ml of reagent QBT (750 mM NaCl, 50 mM MOPS, 15% conc. EtOH, pH 7.0, and 0.15% Triton X-100), and the column was allowed to empty by gravity flow. The supernatant was applied to the "QIAGEN-tip 500" column allowing it to enter the resin by gravity flow. The column was washed twice with 30 ml of reagent QC (1 M NaCl, 50 mM MOPS, and 15% conc. EtOH, pH 7.0), and the plasmid DNA was eluted with 15 ml of reagent QF (1.25 M NaCl, 50 mM Tris-HCl, and 15% conc. EtOH, pH 8.5). Then, the plasmid DNA was precipitated with approximately 0.7 vol. of isopropanol (previously equilibrated to room temperature); the sample was centrifuged immediately at 15000 xg at 4 ^{o}C for 30 min. The supernatant was carefully removed and discarded. The precipitate was washed with 15 ml of cold 70% conc. EtOH, and was dried by air for 5 min. The precipitate was redissolved in a small amount of TE buffer. The amount of plasmid DNA prepared was determined by DNA fluorometry (Model TKO 100). A test of the identity of isolated plasmid DNA was performed by restriction digestion with EcoRI for the alcohol dehydrogenase (ADH) gene, HindIII for the α-tubulin gene, and PvuII for the 180-bp centromeric repetitive fragment.

Restriction Digestion and Southern Blotting of Plant DNA

The DNA extracted from leaf tissues of both CaMV-infected and healthy A. thaliana was digested by a methylation-sensitive restriction enzyme, XhoI, SalI, HpaII, ClaI, or HhaI, or by a methylation-insensitive restriction enzyme EcoRI. For each digestion, 3 μg of plant DNA, 2 μl of the restriction enzyme and 1/10 of total volume of the 10x enzyme buffer were used and mixed well in an amount of water to make 30 μl total. The digestions were carried out at 37 ^{o}C for 3-4 hours. After digestion, 5 μl of dye solution was added into each tube. Then, the samples were loaded on a 1% agarose gel for electrophoresis at 70 V (5.6 V/cm) for 1.5 hours using Loening buffer (0.036 M Tris base, 0.03 M NaH_2PO_4, and 0.001 M EDTA, pH 7.8). After electrophoresis, the DNAs were denatured

and neutralized, following a standard protocol (Maniatis et al., 1987). Then, the separated DNA fragments were transferred by capillary action from the agarose gel to a nylon membrane by Southern blotting using 20x SSC buffer (3 M NaCl and 0.3 M sodium citrate) as a diffusion solution. Then, the DNAs were fixed on the nylon membrane by UV-crosslinking.

Nick Translation (Preparation of Radioactive Probes)

In a 1.5 ml Eppendorf tube, 1 µl of 10x H buffer (66 mM Tris-HCl, pH 7.4, 66 mM $MgCl_2$, 10 mM dithiothreitol and 0.5 M KCl), 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dTTP, and ^{32}P-dCTP, and 0.1 µg of one of the plasmid DNAs prepared were mixed with 3.5 µl water and 1 µl of 10^{-5} µg/µl DNase I solution; the tube was incubated at room temperature for 1 min. and kept on ice. Then, 0.33 µl of DNA polymerase holoenzyme (2 units) was added, and the tube was incubated at 15 °C in a Neslab water bath for 3 hours. The DNA sample was applied to a commerical G-50 Sephadex spin column "Quick Spin-TM column (TE)" for purifying the radioactively-labeled plasmid DNA through centrifugation at 1100 xg for 4 min.

Hybridization of Nylon Sheets with Radioactive DNA

The nylon membrane carrying the plant DNA fragments was wetted with 20 ml of 4x SSC solution. Then, the membrane was incubated with 10 ml of the prehybridization solution (200 mg/L calf thymus DNA, 50 mM sodium phosphate, pH 7.0, 5x Denhardt's solution and 5x SSC solution) in a 65 °C water bath for 2 hours (50x Denhardt's solution contained 1% polyvinylpyrrolidone, 1% Ficoll and 1% bovine serum albumin). The membrane was transfered into 5 ml of the hybridization solution (100 mg/L calf thymus DNA, 20 mM sodium phosphate, pH 7.0, 1x Denhardt's solution, 1x SSC solution, plus the radioactively-labeled plasmid DNA prepared), and was incubated in a 65 °C water bath for hybridization for 17.5 hours. Then, the hybridized membrane was washed with 2x

SSC solution for 30 min. twice, and 0.1x SSC solution (preheated to 65 °C) for 15 min. twice, wherein both the SSC solutions contained 0.1% SDS. Finally, the radioactively-labeled nylon membrane was exposed to an X ray-film for autoradiography about one week.

Rehybridization of Nylon Sheets with Radioactive DNA

The previously-bound radioactive probe on the nylon membrane was removed by boiling the membrane in 15 ml of the TE-SDS buffer (10 mM Tris-HCl, pH 7.0, 1 mM EDTA and 0.1% SDS) for 30 min. The membrane was washed with shaking in the 2x SSC/0.1% SDS solution for 5 min. Efficiency of stripping the bound radioactive probe was assessed by exposing the stripped membrane to a Geiger-Mueller monitor. Then, another radioactive probe was hybridized to the stripped membrane for completing another cycle of the hybridization experiment.

CHAPTER IV

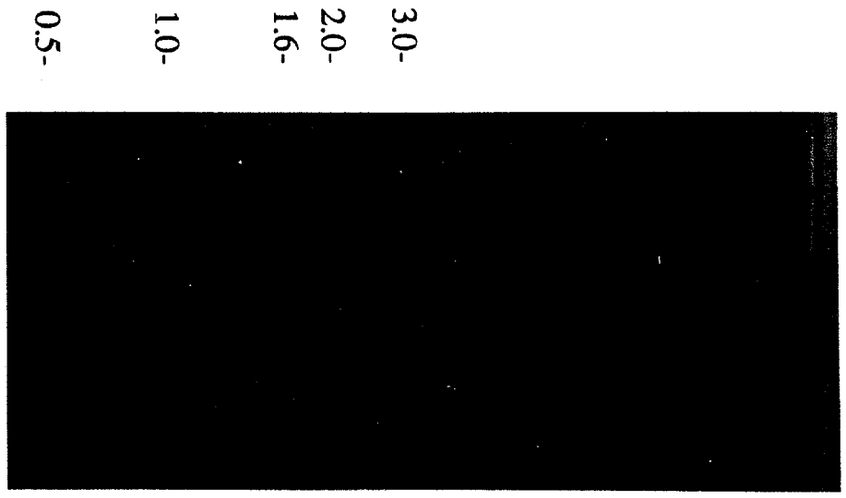
RESULTS

The disease symptoms appearing on the CaMV-infected rosette leaves of A. thaliana harvested in this study included systemic vein clearing and chlorotic spots, which were the same as described in the published papers (Balazs and Lebeurier, 1981; Melcher, 1989). In addition, the symptoms of mottled chlorotic mosaic and fully chlorotic spots appeared on the cauline leaves. Though some cells of A. thaliana leaves may have been CaMV-infected, those leaves without an appearance of visible disease symptoms (especially for old leaves) were not harvested. Further, Maule et al. (1983) indicated that for turnip leaves showing full systemic symptoms, 100% of the mesophyll cells contain CaMV. As a result, only those A. thaliana leaves with the visible disease symptoms were harvested for plant DNA extraction.

Plasmid DNAs containing part of the A. thaliana ADH gene, part of the A. thaliana α-tubulin gene, or the A. thaliana 180-bp centromeric repetitive fragment were selected at random for preparation of a radioactive probe and were available in our laboratory. A test of the identity of isolated plasmid DNAs was performed by restriction digestion and gel electrophoresis (Figure 1). For plasmid pGEMadh1 containing part of the A. thaliana ADH gene, EcoRI restriction digestion cut the G/AATTC sequence motifs, and then gel electrophoresis produced three fragments of 3.1 kb, 2.6 kb and 0.7 kb, as expected. Similarly, for plasmid pSRL51 containing part of the A. thaliana α-tubulin gene, restriction digestion with HindIII cut the A/AGCTT sequence motifs, and gel electrophoresis

Figure 1. Restriction Digestion of Plasmid DNAs.

One μg of plasmid DNA containing the Arabidopsis thaliana alcohol dehydrogenase (ADH) gene, alpha-tubulin gene or 180-bp centromeric repeat fragment was respectively digested with a restriction enzyme EcoRI, HindIII, or PvuII. The digested plasmid DNAs were electrophoresed on a 1% agarose gel at 70 V (5.6 V/cm) for 1.5 hours. The gel was then stained with 1 μg/ml ethidium bromide. The sizes (Kb) of selected bands of the DNA size standards (1 Kb ladder) are shown.



1 Kb ladder standard

ADH gene digested with EcoRI

Tubulin gene digested with HindIII

180-bp repeat digested with PvuII

Figure 2. Restriction Digestion of Healthy and CaMV- Infected Arabidopsis thaliana DNAs.

Three μg of A. thaliana DNA isolated from healthy or CaMV-infected A. thaliana was digested with a methylation-insensitive restriction enzyme EcoRI, or a methylation-sensitive restriction enzyme XhoI, SalI, HpaII, ClaI, or HhaI, and was electrophoresed on a 1% agarose gel at 70 V (5.6 V/cm) for 1.5 hours. The gel was then stained with 1 μg/ml ethidium bromide. The symbol " + " means that these lanes were loaded with CaMV-infected A. thaliana DNA; the symbol " - " means that these lanes were loaded with healthy A. thaliana DNA. The clear bands on the CaMV-infected lanes were the restriction fragments of CM4-184 viral DNA.

EcoRI
XhoI
SalI
HpaII
ClaI
HhaI
Infection
- + - + - + - + - + - +

infected plant DNAs showed a similar intensity between the healthy lanes and CaMV-infected lanes (data not shown). At first, the problem was considered due to incomplete restriction digestion of the plant DNAs, such that a lot of large plant DNA fragments were produced and the large DNA fragments could not be separated well by gel electrophoresis. Therefore, in the extraction procedure of A. thaliana DNA, two more steps were added: (1) phenol extraction to remove proteins so as to make sure that no proteins remained in the plant DNA samples, (2) RNase A treatment to destroy RNA. In addition, I found that for the same DNA sample, the values of DNA determination by DNA fluorometry or spectrophotometry were not identical; the value determined by DNA spectrophotometry was always at least 2-3 fold greater than that by DNA fluorometry. Therefore, one of the possiblities to account for the difference in intensity on the electrophoresis gel was that more than the estimated μg of the CaMV-infected plant DNA was loaded. In particular, for the CaMV-infected plant DNA, the value of DNA determination by DNA fluorometry may have been lower than the true amount of DNA. It was hypothesized that at least one substance may exist in the samples of CaMV-infected plant DNA that resulted in a quenching effect to lower the apparent DNA level during the DNA determination. Alternatively, the other possibility was that less than the estimated μg of healthy plant DNA was actually loaded. This event may be explained due to the existence of a UV-absorbing contaminant in the sample of healthy plant DNA rather than in the sample of CaMV-infected plant DNA. As a result, the value of DNA determination for healthy plant DNA by DNA spectrophotometry was higher than that it really was. Thereafter, based on the different values of DNA determination, two sets of experiments including the extra two purification steps were performed. At this time, the apparent 2 μg rather than 3 μg of healthy and CaMV-infected A. thaliana DNAs was loaded on each gel lane. (This change could also facilitate the transfer of plant DNAs from a gel to a nylon membrane by Southern blotting.) However, after electrophoresis, for these two sets of experiments, a heterogeneous distribution on electrophoresis gels of the restriction-digested plant DNAs similar to that

shown on Figure 2 was obtained. As a result, I could exclude the possibility that the incomplete restriction digestion may have occurred. In addition, the following hybridization results were obtained based on the DNA determination by DNA fluorometry.

The hybridization of restriction-digested healthy and CaMV-infected A. thaliana DNAs with part of the A. thaliana ADH gene resulted in no differences in the band patterns between the healthy and CaMV-infected plant DNAs shown on the autoradiograph (Figure 3). For this hybridization, I transferred to a nylon membrane the restriction-digested plant DNAs from the electrophoresis gel that showed a similar intensity of the heterogeneous distribution between healthy and CaMV-infected plant DNAs, so that the intensity of band patterns in the CaMV-infected lanes on the autoradiograph was similar to that in the healthy lanes. In addition, there is, for example, one SalI restriction site in the A. thaliana ADH gene, and thus two SalI-digested hybridization bands were shown on Figure 3, as expected. Also, I expected that two EcoRI-digested hybridization bands should have shown on Figure 3; however, no EcoRI-digested hybridization bands appeared on Figure 3. Nevertheless, an appearance of pairs of bands on the healthy and CaMV-infected lanes on Figure 3 indicated that for these methylation sites examined, CaMV infection did not alter the methylation status of cytosine residues in the A. thaliana DNA probed by this A. thaliana ADH gene. Similarly, hybridization of the restriction-digested plant DNAs with part of the A. thaliana α-tubulin gene probed showed no differences between the healthy and CaMV-infected plant DNAs in band patterns on the autoradiograph (Figure 4). Though the intensity of band patterns in the CaMV-infected lanes was greater than that in the healthy lanes, these differences in intensity were consistent with those shown on Figure 2. Further, more hybridization bands appeared on Figure 4 than I expected; for example, there was no SalI restriction site in the A. thaliana α-tubulin gene, and thus I expected that only one hybridization band rather than two appeared on Figure 4. As a result, one of the possibilities to explain the difference in the number of hybridization bands was that

Figure 3. Hybridization of Restriction-Digested Fragments of Healthy and CaMV-Infected Arabidopsis thaliana DNA with the A. thaliana Alcohol Dehydrogenase (ADH) Gene.

Three μg of A. thaliana DNA isolated from healthy or CaMV-infected A. thaliana was digested with a methylation-insensitive restriction enzyme EcoRI, or a methylation-sensitive restriction enzyme XhoI, SalI, HpaII, ClaI, or HhaI, and was electrophoresed on a 1% agarose gel at 70 V (5.6 V/cm) for 1.5 hours. The plant DNAs on the gel were transfered to a nylon membrane by Southern blotting. The membrane was then hybridized with the cloned A. thaliana ADH gene. The symbol " + " means that these lanes were loaded with CaMV-infected A. thaliana DNA; the symbol " - " means that these lanes were loaded with healthy A. thaliana DNA. The autoradiograph indicated no differences in the methylation band patterns of the fragments between healthy and CaMV-infected A. thaliana DNAs.

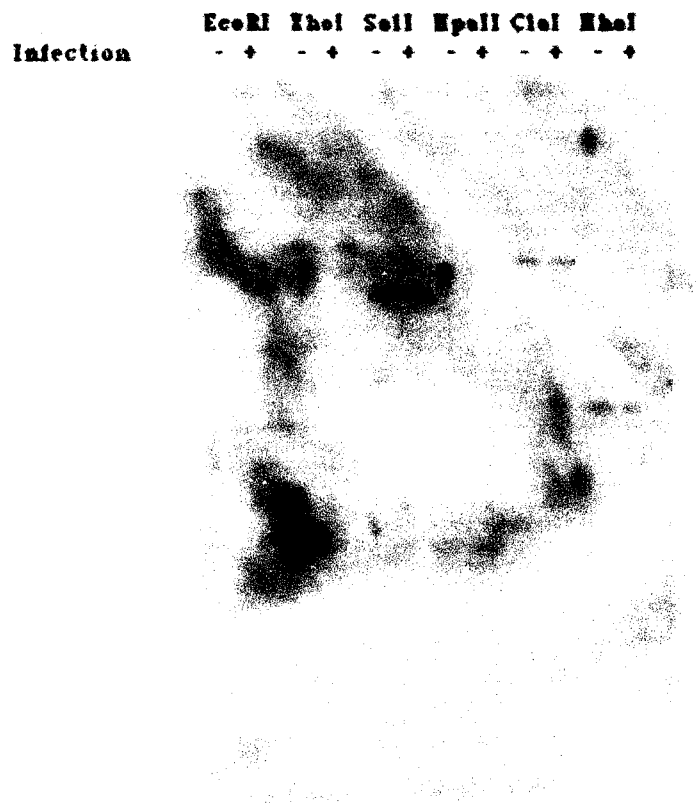
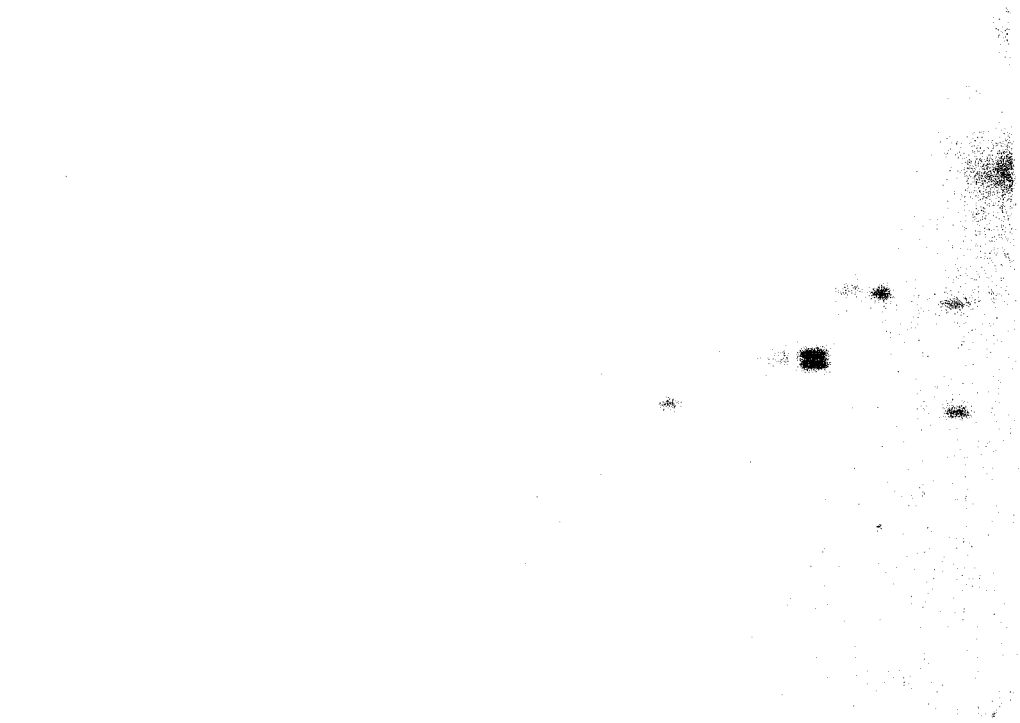

EcoRI XhoI SalI HpaII ClaI HhaI
Infection
- + - + - + - + - + - +

Figure 4. Hybridization of Restriction-Digested Fragments of Healthy and CaMV-Infected Arabidopsis thaliana DNA with the A. thaliana alpha-Tubulin Gene.

Three μg of A. thaliana DNA isolated from healthy or CaMV-infected A. thaliana was digested with a methylation-insensitive restriction enzyme EcoRI, or a methylation-sensitive restriction enzyme XhoI, SalI, HpaII, ClaI, or HhaI, and was electrophoresed on a 1% agarose gel at 70 V (5.6 V/cm) for 1.5 hours. The plant DNAs on the gel were transfered to a nylon membrane by Southern blotting. The membrane was then hybridized with the cloned A. thaliana alpha-tubulin gene. The symbol " + " means that these lanes were loaded with CaMV-infected A. thaliana DNA; the symbol " - " means that these lanes were loaded with healthy A. thaliana DNA. In comparison with the electrophoresis gel shown on Figure 2, the autoradiograph indicated no significant differences in the methylation band patterns of the fragments between healthy and CaMV-infected A. thaliana DNAs.

Infection	EcoRI		XhoI		SalI		HpaII		ClaI		HhaI	
	-	+	-	+	-	+	-	+	-	+	-	+

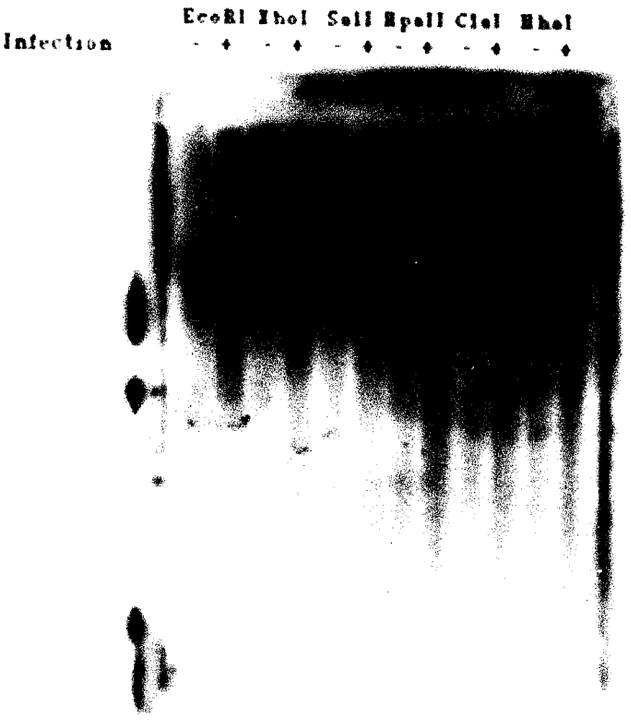


there are at least four α-tubulin gene and/or pseudogene families in the A. thaliana genome (Ludwig et al., 1987), such that some other homologous A. thaliana α-tubulin gene fragments could hybridize the radioactive α-tubulin gene probe. Furthermore, the intensity of one of the ClaI-digested hybridization bands shown on Figure 4 was greater on healthy lane than on CaMV-infected lane. Though, due to a different preparation of plant DNA, this result did not show up on the first hybridization autoradiograph probed by the A. thaliana α-tubulin gene, the inverse intensity on the ClaI-digested band may possibly imply a specific change of cytosine methylation in 5' upstream region of the A. thaliana α-tubulin gene, which needs to be further investigated. Nevertheless, an appearance of pairs of bands on the healthy and CaMV-infected lanes on Figure 4 indicated that for other methylation sites examined, CaMV infection did not alter the methylation status of cytosine residues in A. thaliana DNA probed by the A. thaliana α-tubulin gene. In addition, the A. thaliana α-tubulin and ADH genes were large DNA fragments such that clear hybridization band patterns appeared on the upper portions of autoradiographs.

The hybridization of healthy and CaMV-infected A. thaliana DNAs with the A. thaliana 180-bp centromeric repeat fragment resulted in a heterogeneous distribution of the reactive DNAs on the autoradiograph (Figure 5), where clear hybridization bands only appeared on the EcoRI-digested lanes. This hybridization result was different from those obtained with the A. thaliana ADH and α-tubulin gene probes. The heterogeneous distribution of reactive DNAs shown on the autoradiograph (Figure 5) was similar to that of wild-type MspI-digested A. thaliana DNA hybridized with the A. thaliana 180-bp centromeric repeat fragment (Vongs et al., 1993). As shown on Figure 5, the intensity of this heterogeneous distribution on the CaMV-infected lanes was greater than that on the healthy lanes. The differences in intensity of heterogeneous distributions on Figure 5 were consistent with those shown on Figure 2. Therefore, for these methylation sites examined, CaMV infection did not alter the methylation status of cytosine residues in the A. thaliana centromeric repetitive arrays probed by the A. thaliana 180-bp repeat fragment.

Figure 5. Hybridization of Restriction-Digested Fragments of Healthy and CaMV-Infected Arabidopsis thaliana DNA with the A. thaliana 180-bp Centromeric Repeat Fragment.

Three μg of A. thaliana DNA isolated from healthy or CaMV-infected A. thaliana was digested with a methylation-insensitive restriction enzyme EcoRI, or a methylation-sensitive restriction enzyme XhoI, SalI, HpaII, ClaI, or HhaI, and was electrophoresed on a 1% agarose gel at 70 V (5.6 V/cm) for 1.5 hours. The plant DNAs on the gel were transfered to a nylon membrane by Southern blotting. The membrane was then hybridized with the cloned A. thaliana 180-bp centromeric repeat fragment. The symbol " + " means that these lanes were loaded with CaMV-infected A. thaliana DNA; the symbol " - " means that these lanes were loaded with healthy A. thaliana DNA. In comparison with the electrophoresis gel shown on Figure 2, the autoradiograph indicated no significant differences in the methylation band patterns of the fragments between healthy and CaMV-infected A. thaliana DNAs.


EcoRI XhoI SalI BpmI ClaI HhaI
Infection
- + - + - + - + - + - +

Though the intensities of the heterogeneous distribution of restriction-digested A. thaliana DNAs between healthy and CaMV-infected lanes shown on Figure 2 were different, incomplete transfer of the large fragments of plant DNA from an agarose gel to a nylon membrane by Southern blotting may have also occurred. Therefore, to address the problem of incomplete transfer of plant DNAs, I soaked the agarose gel containing the restriction-digested 2 μg of the plant DNAs, after gel electrophoresis, in 0.25 N HCl solution for 10 min. to break down the large plant DNA fragments into small DNA fragments so as to facilitate the complete transfer of plant DNAs. In addition, twice the amount of 20x SSC buffer was used to elongate the process of DNA transfer from the agarose gel to the nylon membrane. Furthermore, after Southern blotting to transfer plant DNAs from the gel to the membrane, I soaked the gel in 1 μg/ml ethidium bromide for 20 min. and then took a photograph of the gel. The photograph showed a complete black background, revealing that no plant DNA was left on the gel. Thereafter, hybridization of the membranes containing the digested fragments of 2 μg plant DNAs with the A. thaliana 180-bp centromeric repeat fragment probe also resulted in a heterogeneous distribution of the reactive DNAs similar to that shown on Figure 5. Thus, I could exclude the possibility that incomplete transfer of plant DNAs from the gel to the nylon membrane may have occurred.

For reproducibility, the hybridization with each of the A. thaliana ADH and α-tubulin genes probed was repeated three times; and, for the A. thaliana 180-bp centromeric repeat fragment probed, to address the problem of incomplete restriction digestion and incomplete transfer of the plant DNAs, the hybridization experiment was repeated nine times.

In summary, there were possibly no significant differences in the methylation patterns of fragments between the healthy and CaMV-infected A. thaliana DNAs shown on the autoradiographs, suggesting that, at least for this virus-host combination and for the genes and sites examined, CaMV infection did not alter the methylation status of the plant DNA,

and thus changes in DNA methylation probably play no role in the generation of disease symptoms.

CHAPTER V

DISCUSSION

Some cytosine residues in particular sequences in plant DNA are susceptible to methylation. This methylation may be associated with regulation of genes. Virus infection has been suggested to alter gene regulation, and this event may reflect changes in the methylation status of genes. In this study, it was hypothesized that changes in cytosine methylation of A. thaliana DNA via CaMV infection were correlated with disease symptoms of the plant. The methylation status of cytosine residues in the restriction sites examined was expected to be changed in the CaMV-infected A. thaliana DNA relative to the healthy A. thaliana DNA. The band patterns on autoradiographs indicated whether or not the methylation patterns of fragments of CaMV-infected A. thaliana DNA differed. The experimental result showed that, for this CaMV-A. thaliana combination and for the genes probed and the restriction sites examined, no significant differences in the methylation patterns of fragments from healthy and CaMV-infected plant DNAs were found, suggesting that CaMV infection did not alter the methylation status of the plant DNA.

This detection of cytosine methylation was restricted to those cytosine residues that lie within the recognition sequence of a restriction enzyme that is sensitive to cytosine methylation. Specific changes in cytosine methylation would not be detected if the cytosine residues concerned did not lie within the recognition sites of the enzymes used. Methylation-sensitive restriction enzyme XhoI digests the sequence CTCGAG, involving a CG motif; similarly, SalI digests GTCGAC, ClaI digests ATCGAT, and HhaI digests CGCG. Another methylation-sensitive restriction enzyme HpaII digests the sequence CCGG, involving both CG and CNG motifs. The methylation status of these CG or CNG

motifs in the restriction sites examined in A. thaliana DNA did not alter in response to CaMV infection. This result did not rule out that specific changes in the cytosine methylation in other sites of the plant DNA could have occurred. It is possible that a subset of methylation-sensitive sites, not assayed by the probes used in this study, is specifically altered by CaMV infection. Nevertheless, as a random selection of the restriction enzymes used and the genes probed in this study, the combinations of these restriction sites and genes examined should be considered as representatives of other genes and sites. Though it could not be ruled out that CaMV infection altered the methylation status of cytosine residues in A. thaliana DNA, the chance of finding the specific changes of cytosine methylation in A. thaliana DNA by Southern blotting-hybridization with methylation-sensitive restriction enzymes is small. In addition, most of the cytosine methylation in A. thaliana DNA was found in the highly repeated DNA fraction, a large portion of which is accounted for by the 180-bp centromeric repetitive sequence and the ribosomal RNA genes (Pruitt and Meyerowitz, 1986), implying that only a few cytosine residues in other functional A. thaliana genes were methylated. Thus, if possible, more fragments of A. thaliana DNA other than these three genes probed and more methylation-sensitive restriction enzymes other than those five used in this study should be examined for detecting specific changes of cytosine methylation in A. thaliana DNA. In addition, the global change in 5-mC content in the A. thaliana genome by CaMV infection should be determined.

As a very limited number of the potential methylation sites in the A. thaliana genome were assayed in this study using methylation-sensitive restriction enzymes, the failure to detect specific changes in cytosine methylation does not mean that cytosine methylation was not involved in gene regulation of A. thaliana or that changes in cytosine methylation play no role in the generation of disease symptoms. Another major technique to study cytosine methylation is genomic sequencing, which can determine the methylation status of every cytosine residue on either strand of the DNA. For further investigation of the specific

changes in cytosine methylation of A. thaliana genome by CaMV infection, a particular gene of A. thaliana , such as the alcohol dehydrogenase (ADH) gene, can be isolated from the CaMV-infected A. thaliana leaf tissues. The nucleotide sequence of the wild-type A. thaliana ADH gene was known (Chang and Meyerowitz, 1986). The genomic sequencing can distinguish between methylated and unmethylated cytosine residues in a native genomic DNA where the sequence was known, so that by comparison with the band patterns on autoradiograph, specific changes in cytosine methylation of the CaMV-infected A. thaliana ADH gene can be determined by this method.

Neither incomplete restriction digestion of the plant DNAs nor incomplete transfer of the plant DNAs from the gel to the nylon membrane could account for the difference in the intensity of a heterogeneous distribution between healthy and CaMV-infected lanes shown on Figures 2 and 5, though the same apparent amount, 3 μg, of restriction-digested plant DNAs was loaded on each gel lane. Consequently, one of the possible explanations for this difference in intensity was that actually more than 3 μg of the CaMV-infected plant DNA was loaded, inferring that the value of DNA determination of CaMV-infected plant DNA by DNA fluorometry was lower than that it really was. A hypothesis was then proposed that CaMV infection was able to induce A. thaliana to produce at least one new substance or to increase the production of already existing substances in the plant cells; as a result, the substance(s) in each sample of the CaMV-infected plant DNA interfered with the DNA determination and resulted in a quenching effect to reduce the DNA estimated level. The existence of this hypothetical substance was not proved. Nevertheless, it may be important to determine which substances are produced or over-produced in the A. thaliana plant cells by CaMV infection. Those substances may be related to the disease symptoms in A. thaliana or to viral replication and spread in the plant cells.

Another possibility to account for the difference in intensity was that less than 3 μg of healthy A. thaliana DNA was actually loaded on each gel lane: the value of DNA determination for healthy plant DNA by DNA spectrophotometry was higher than the true

amount of DNA. A hypothesis to give an explanation was that a UV-absorbing contaminant may exist in the sample of healthy plant DNA rather than in that of CaMV-infected plant DNA, and thus could interfere with the DNA determination so as to raise the DNA estimated value.

The experimental result published by Vongs et al. (1993) can indirectly support that, for the methylation sites examined by HpaII restriction digestion, CaMV infection did not alter the methylation patterns of fragments of A. thaliana DNA probed by the A. thaliana 180-bp repeat fragment. Three A. thaliana DNA hypomethylation ddm1 mutants containing hypomethylated centromeric repetitive DNA arrays susceptible to the digestion of a methylation-sensitive restriction enzyme HpaII or MspI were isolated. The nylon membrane carrying the HpaII-digested fragments of both wild-type A. thaliana DNA and the hypomethylation ddm1 mutant DNAs was hybridized with the A. thaliana 180-bp repeat fragment. A heterogeneous distribution of the reactive DNAs with clear band patterns shown on the lower portion of autoradiographs indicated that the HpaII-digested CCGG motifs in the centromeric repetitive arrays of A. thaliana plant genome were hypomethylated in these hypomethylation DNA ddm1 mutants. It was expected that these clear band patterns, for the HpaII restriction site examined and for the 180 bp repeat fragment probed, should also occur on the lower portion of autoradiograph shown on Figure 5, if CaMV infection really altered the methylation status of cytosine residues in the A. thaliana centromeric repetitive genes. However, these clear band patterns did not occur on Figure 5. Thus, CaMV infection did not alter the cytosine methylation in the A. thaliana centromeric repetitive DNA sequences.

In summary, I can conclude that for this CaMV-A. thaliana combination and for the genes and the restriction sites examined, no changes in cytosine methylation for the generation of disease symptoms of the plant were found. Nevertheless, I can not conclude that specific changes in cytosine methylation in the A. thaliana plant genome were not correlated with the generation of disease symptoms. Consequently, further investigations

are needed so as to understand the relationship between cytosine methylation and plant diseases.

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